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(57) Abstract

Protein-free cell culture media supplements are described consisting of synergistic combinations of medium components, which when added to cell culture media, either serum supplemented or serum-free, enhance cell growth, culture longevity and product expression. Characteristic components of the supplement are glutamine or glutamate, tryptophan and phospholipid precursors, e.g. cholin and ethanolamine.

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CELL CULTURE MEDIUM FOR ENHANCED CELL GROWTH, CULTURE LONGEVITY AND PRODUCT EXPRESSION

This invention is in the general field of animal cell culture. More particularly, the invention concerns improved media for the cultivation of animal cells and the production of natural and recombinant products derived therefrom.

To date, efforts have been undertaken to develop culture conditions to maximize cell culture growth and thereby increase resultant product yield. Early work in the development of animal cell culture media focused on the formulation of such media to achieve rapid cell proliferation (White, P.R., 1946, Growth, 10:231-289, and Waymouth, C., 1974, J. Natl. Cancer Inst., 53:1443-1448). Such media incorporate specific nutrients especially sugars, amino acids, vitamins, salts, and in some cases trace metal ions, purines, and pyrimidines. These media are most often supplemented with animal serum. Today some of the more widely used basal media for mammalian cell cultures include Hams F-12, Dulbecco's modified Eagle's medium (DME), RPMI 1640, and Iscove's modified DME.

Production of human monoclonal antibodies using hybridoma cell lines will be used in this application as an example for product expression in cell culture.

Culture media have been previously described which were developed specifically for low serum and serum-free mammalian cell cultures for production of monoclonal antibodies. One such serum-free medium is disclosed in European Patent Publication 076,647, published April 13, 1983. Other media have been developed by changing levels of supplements such as trace elements, and vitamins and incorporating purified protein hormone additives. References to such media include, for example, Barnes, D., et al., 1980 Cell, 22:649-655; Cleveland, W.L., et al. 1983, J. Immunol. Meth., 56:221-234; Iscove, N., et al., 1978, J. Exp. Med., 147:923-933; Kawamoto, T., et al., 1983, Analytical Biochemistry, 130:445-453; Kovar, J., et al., 1984, Immunology Letters, 7:339-345; Murakami, H., et al., 1983, Argic. Biol. Chem., 47(8):1835-1840; Murakami, H., et al., 1982, Proc. Natl. Acad. Sci. USA, 79:1158-1162; Muzik, H., et al., 1982, In Vitro, 18:515-524; and Wolpe, S.D., "In Vitro Immunization and Growth of Hybridomas in Serum-Free Medium", in J.P. Mather, ed., Mammalian Cell Culture, Plenum Press, New York, 1984; Hagiwara, H., et al., 1985, 117-122 in H. Murakami et al. (eds) Growth and Differentiation of Cells in Defined Environment, Springer-Verlag, Berlin, 1985; Tharakan, J.P., et al., 1986, J.

Immunol. Meth., 94:225-235; Cole, S.P.C., 1987, J. Immunol. Meth., 97:29-35; McHugh, Y.E., 1983, BioTechniques, June/July issue:72-77. Components which are common to most if not all these media include glucose at concentrations up to 4.5 g/L, glutamine at concentrations of 2-4 mM, choline generally at about 1-4 mg/L, tryptophan and other amino acids. Tryptophan is generally present at concentrations less than 20 mg/L. Several of these media also contain the growth factors insulin and transferrin.

Efforts to increase antibody yield have focused primarily on means to optimize cell growth and cell density. As a general point of reference, antibody titres from murine hybridoma cell lines are highly variable from cell line to cell line and range typically from 10 to 350 mg/L (Lambert, K.J., et al., 1987, Dev. Indust. Microbiol. 27:101-106). Human monoclonal antibody expression from human/human or human/mouse fusions are also highly variable from cell line to cell line and range typically from 0.1 to 25 mg/L (Hubbard, R., Topics in Enzyme and Fermentation Biotechnology, Chapt. 7:196-263, Wisemand, A., ed., John Wiley & Sons, New York (1983). These values are indicative of culture conditions that are optimized for cell growth.

Another approach from the literature to increasing product production is to achieve high cell densities by cell recycle or entrapment methods. Examples of these methods include hollow fiber reactors (Altshuler, G.L., et al., 1986, Biotech. Bioeng., XXVIII:646-658; ceramic matrix reactors (Marcipar, A., et al., 1983, Annals. N.Y. Acad. Sci., 413:416-420; Nature, 302:629-630); perfusion reactors (Feder, J., et al., 1985, American Biotech. Laboratory, III:24-36) and others.

While a variety of methods to increase product expression from cell culture are being explored, the primary focus is still on the optimization of cell growth. In typical culture media the culture dies rapidly after maximum cell density is reached.

Another example from the literature documents that, at least for some cell lines, product (monoclonal antibody) production proceeds even after a culture stops growing (Velez, D., et al., 1986, J. Immunol. Meth., 86:45-52; Reuveny, S., et al., 1986, ibid at 53-59). Arathoon, W., et al., 1986, Science, 232:1390-1395 reported that a 1,000 liter hybridoma fermentation produced about 80 grams of monoclonal antibody during the growth phase and another 170 grams of antibody during an extended stationary/death phase. J. Birch, et al., (European Patent Application No.

87/00195, 1987) describe a procedure of Fed-batch culture wherein nutrients are added to a culture over time and culture longevity is increased. Final antibody titres from the culture are thus increased.

Thus, it will be appreciated that there is a critical need for media that will support the growth of animal cells and stimulate the production of products, including antibodies, and other natural or recombinant protein products to greater levels than can be realized using media that are currently available.

Accordingly, the invention presented herein describes a protein-free supplement (the "Primary Supplement"), which when added to standard cell culture media, enhances cell growth, culture longevity and product expression. Examples are given showing that this supplement is particularly effective in production of antibodies using hybridoma cell lines in serum-free culture, where final antibody titre is increased in one example from 80 mg/L to over 250 mg/L. The Primary Supplement is a hitherto unrecognized synergistic combination of standard medium components which are beneficial when added in the prescribed combination, and consist of 1) glutamine, 2) phospholipid precursors, including minimally both choline and ethanolamine, 3) tryptophan, and 4) additional amino acids as required for a particular cell line and product to be produced. When added individually to standard media, these components have little effect, but are extremely effective when added together in the prescribed combination, and the deletion of any of the prescribed components diminishes the desired effect). Several of the components in the Primary Supplement are added to concentrations hitherto considered to be unnecessary or inhibitory. When added together in the prescribed combination, the supplement enhances cell growth, increases culture longevity by maintaining cells in a pseudo-stationary phase wherein product expression continues, and thus results in a significant increase in final product titre.

A second aspect of the invention is a description of several additional components, (the "Class I reagents"), which when added individually, or in various combinations to the Primary Supplement result in a further improvement to culture growth and/or product expression. Included in this class of additional components are reducing agents, trace metal ions, and/or vitamins.

A third aspect of the invention is the formulation of serum-free and serum supplemented media containing the supplement, as well as in media supplemented

with lipids and can be used with the addition of agents to induce solute stress.

A further aspect of the invention is a method of growing cells employing the media supplements described herein such that they can be included in medium at the start of culture, or can be added in a fed-batch or in a continuous manner. The resulting media can be used in various cultivation methods including, but not limited to, batch, fed-batch, hemostat and perfusion, and with various cell culture hardware including, but not limited to, stationary flasks, agitated flasks, spinner flasks, stirred fermentors, airlift fermentors, membrane reactors (including hollow fiber, flat membrane plate, external loop perfusion), reactors with cells retained on a solid support of immobilized/entrapped as in microporous beads, and any other configuration appropriate for the growth or maintenance of the desired cell line.

Figure 1 shows the growth properties and levels of antibody produced by D234 in a typical commercially available media, Ventrex HL-1.

Figure 2 shows the growth properties and levels of antibody produced by D234 in a standard serum free medium composition representative of compositions described in the cell culture literature.

Figure 3 shows the growth properties and levels of antibody produced by D234 in media supplemented with reagents of the Primary Supplement.

Figure 4 shows the growth properties and levels of antibody produced by D234 in media supplemented with reagents of the Primary Supplement and a Class I reagent.

Figure 5 shows the growth properties and levels of antibody produced by D234 in media supplemented with reagents of the Primary and Class I Supplement and used in combination with lipid supplementation.

Figure 6 shows the hybridoma D234 grown in Fed-batch with media supplemented with reagents of the Primary and Class I Supplements and lipids.

The following reference is referred to throughout this application, and is presented here for the convenience of the reader. Ian Freshney (Culture of Animal Cells -- A Manual of Basic Technique, Alan R. Liss, NY, 1987) tabulates compositions of typical basal media for use with serum (Table 7.5:74-75) and typical serum-free media compositions (Table 7.6:76-78) described in the literature for culture of a wide range of animal cells and expression of products therefrom. These tables are incorporated by reference.

The invention described herein is first concerned with a protein-free supplement compatible with standard cell culture media to enhance cell growth, culture longevity and product expression. The Primary Supplement is favored, and has advantages that arise, at least in part, by a hitherto unrecognized synergistic interaction of the reagents employed. Iscove (N.N. Iscove, Culture of Lymphocytes and Hemopoietic Cells in Serum-Free Medium:169-185 in Methods for Serum-Free Culture of Neuronal and Lymphoid Cells) describes the standard method for optimizing cell culture media wherein "the effect of individually doubling and quadrupling the concentration of each component was examined ...". This approach has been widely used in the cell culture field, which fails to recognize the possibility that components may act synergistically and that it may be necessary to combine supplements of several components to see a desired effect.

Components selected from a second group of cell culture reagents (the Class I reagents) when combined with the Primary Supplement can synergize to produce a more favorable cell culture supplement having especially beneficial effects on cells grown to high densities.

Before a detailed description of the classes of reagents is presented, and the media supplements that can be formulated therefrom, a brief definition of some of the technical terms used throughout this application will facilitate understanding the nature of the invention.

Definitions

"Basal medium" is defined herein to include a nutrient mixture of inorganic salts, sugars, amino acids, and, optionally, vitamins, organic acids and/or buffers or other well known cell culture nutrients. These reagents are necessary to support cell growth and reproduction. The preferred basal media which is a component of the instant cell growth media compositions contains neither serum, nor proteins. A wide variety of commercially available basal media are well known to those skilled in the art, and include Dulbeccos' Modified Eagles Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI), Iscove modified Dulbeccos' medium and Hams medium.

The term "hybridoma", refers to a hybrid cell line produced by the fusion of an immortal cell line of immunologic origin, and an antibody producing cell. The

term is meant to include progeny of heterohybrid myeloma fusions, which are the result of a fusion with human cells and a murine myeloma cell line subsequently fused with a plasma cell, referred to in the art as a trioma cell line. Additionally, the term is meant to encompass virtually any immortalized hybrid cell line which produced antibody such as, for example, quadromas. Milstein, C., et al., 1983, Nature, 537:3053. Moreover, the hybrid cell lines can be of virtually any species, including human and mouse.

The term "microemulsion" refers to a lipid mixture emulsified substantially without the aid of proteinaceous materials such as those found in serum, particularly serum albumin. Generally, emulsifiers described in PCT Application No. WO 89/01027, published January 9, 1989, will be employed to form the microemulsion. This patent application is hereby incorporated by reference in its entirety. Generally, pluronic polyols are favored as the emulsifier.

As used herein the term "solute stress" refers to the addition of solutes in such concentrations that produce a growth inhibitory effect or reduced final cell density, that is, a growth rate or maximum cell density less than that determined for optimal growth. However, the level of product expressed at this reduced growth level is comparatively greater than that level of expression achieved at the optimal growth rate owing to an increase in specific (per cell) product expression rate or an increase in longevity of the culture. Generally, solutes and methods described PCT Application No. WO 89/04867, published June 1, 1989, will be employed. This application is hereby incorporated by reference in its entirety.

"Pseudo-Stationary phase" refers to a period of culture growth occurring after the exponential growth phase and wherein the rates of cell growth and death are similar such that the viable cell concentration changes only relatively slowly with time (as compared to during the exponential growth or the death phase).

"Cell growth hormones" or "growth factors" is meant to encompass a large number of molecules either naturally occurring or genetically engineered, as well as fragments, derivatives or modifications thereof, that stimulate the growth, or supports the maintenance of cells in defined media. Examples of the former includes transferrin and insulin, among others. Examples of growth factors includes nerve growth factor, epidermal growth factor, fibroblast growth factor, and endothelial cell growth factor, among others. It is important to note that different animal cell lines

may vary in their requirements for one or more of these molecules, and that the optimal hormones or growth factors, or a mixture of the same, is readily determined using standard cell culture techniques.

The instant invention presents cell culture media supplements that are compatible with the general growth and maintenance requirements of animal (especially mammalian) cells in vitro, and particularly enhances or supports the expression of differentiated properties associated with specific cell types, for example, an elevated production of monoclonal antibodies by hybridoma cell lines. Thus, it will be appreciated that the instant supplements have widespread applications, being generally useful for routine culture of cells, as well as being employed in those instances when great amounts of a natural or recombinant cellular product is desired that is produced by cells in culture.

Primary Supplement

The Primary Supplement of the current invention consist of a class of reagents that includes, in combination, 1) glutamine, 2) phospholipid precursors including preferably choline and ethanolamine, 3) tryptophan, and 4) additional amino acids as required for the particular cells lines.

a) Glutamine:

In the Primary Supplement, glutamine is included at over 5 mM, and preferably at 8-20 mM. It is important to note that these concentrations are significantly above those typically found in cell culture media.

Glutamine is recognized as both an amino acid building block for protein synthesis and as a primary energy source in cell culture (W.L. McKeehan, Glycolysis, Glutaminolysis and Cell Proliferation, Cell Biology Intro. Reports, 1982, 6(7):635-650, L.J. Reitzer, et al., Evidence that Glutamine, Not Sugar, Is the Major Energy Source for Cultured HeLa Cells, J. Biological Chemistry, 1979, 254(B):2669-2676, H.R. Zielke, et al., Reciprocal Regulation of Glucose and Glutamine Utilization by Cultured Human Diploid Fibroblasts, J. Cell Physiol., 1978, 95:41-48.

Reference to the summary of typical media compositions tabulated in Freshney, above, shows that glutamine is typically included at 2 or 4 mM in standard serum-supplemented and serum-free medium formulations. In contrast, as mentioned

above, the composition of the instant invention contains elevated amounts of glutamine above 5 mM, and preferably about 8-20 mM.

Both glutamine metabolism as well as spontaneous decomposition of glutamine (G.L. Trisch, et al., Spontaneous Decomposition of Glutamine in Cell Culture Media, Experimental Cell Research, 1962, 28:360-364) result in the release of ammonium ion which is widely described in the literature as toxic to either cell growth or protein production (S. Reuveny, et al., Factors Affecting Cell Growth and Monoclonal Antibody Production in Stirred Reactors, J. Immunl. Methods, 1986, 86:53-59, and some researchers have argued that glutamine when added at high concentrations will have a toxic effect (indirectly through increased production of ammonium ion). Some researchers have advocated minimizing the concentration of glutamine present in the culture by adapting the culture to grow in the absence of glutamine and with glutamic acid as an alternate substrate (J.B. Griffiths, et al., The Uptake of Amino Acids by Mouse Cells (Strn LS) During Growth in Batch Culture and Hemostat Culture, The Influence of Cell Growth Rate, Proc. Roy Soc. London , 1967, 168:421-438), or by utilizing slow addition of glutamine throughout the time course of the culture to maintain a relatively constant low concentration of glutamine (M.W. Glacken, et al., Reduction of Waste Product Excretion via Nutrient Control: Possible Strategies for Maximizing Product and Cell Yields on Serum in Cultures of Mammalian Cells, Biotechnol. Bioeng., 1986, 28:1376-1389).

Based on the forgoing discussion, it will be realized that for cell lines which have been adapted to grow with glutamate or other substitute for glutamine, that the methods of the current invention are still applicable, except that elevated levels of glutamate or other substitute would be included in the supplement in place of glutamine. For cell lines that have been adapted to grow with low concentrations of glutamine maintained by glutamine addition throughout the culture, the methods of the current invention are also applicable except that the increased quantity of glutamine will be added gradually over the course of the culture.

Recently, some researchers have found that addition of supplemental glutamine late in culture can increase culture longevity for some cell lines (S. Reuveny, et al., Factors Affecting Cell Growth and Monoclonal Antibody Production in Stirred Reactors, J. Immunol. Methods, 1986, 86:53-590).

We have found that supplemental glutamine can be added either at the start

of culture, or during the course of culture with the total of glutamine added to about 5-40 mmoles and preferably about 8-20 mmoles per liter. We believe that late in culture, in a post exponential growth pseudo-stationary phase, that glutamine can be a major energy source for the cells, (i.e. glutamine consumption continues while glucose consumption may decline). We find that glutamine supplementation is necessary, but not sufficient to achieve the desired increase in culture longevity. To achieve the optimal performance, glutamine must be added in combination with the other reagents of the Primary Supplement.

b) Phospholipid Precursors:

The Primary Supplement also contains phospholipid precursors, selected from the group including but not limited to, serine, inositol, choline, ethanolamine, and glycerol. While these components are all phospholipid precursors, they also have other biochemical roles, and this invention should not be considered as being limited by any proposed hypothesis of a mechanism of action.

Serine is considered an essential amino acid, and inositol an essential vitamin. Most cell culture media contain serine and inositol at adequate levels for their roles as phospholipid precursors and other biochemical roles.

Choline is included as a vitamin in most media at 1-4 mg/L. However, on occasion, choline has been used at higher concentrations. For instance, to grow cells of nonlymphoid origin, Ham's (R.G. Ham, Clonal Growth of Mammalian Cells in a Chemically Defined Synthetic Medium, Proc. Natl. Acad. Sci. USA, 1965, 53:288-293) includes choline at 15 mg/L in a serum-free medium for clonal growth of Chines Hamster Ovary Cells. J.Birch, et al., J. Cell Sci., 1969, 5:135-142) includes choline at 20 mg/L in a serum containing medium for growth of mouse fibroblasts. Waymouth's (C. Waymouth, Rapid Proliferation of Sublines of NCTC Clone 929 Mouse Cells in a Simple Chemically Defined Medium, J. Natl. Cancer Inst., 1959, 22:1003-1017) medium MB 752/1 for culture of mouse L929 fibroblast connective tissue cell line is exceptional in including choline at 250 mg/L. Although Ham, Birch and Waymouth developed media to support cell growth, they did not study the production of biological products produced by cells grown in the media. We have found that cells, preferably antibody secreting cells, grow and secrete maximal amounts of antibody in media containing choline supplemented to a level of greater

than about 4 mg/L, and preferably at approximately 4-40 mg/L in combination with the other reagents of the Primary Supplement. At these concentrations, choline does not become limiting and is without apparent toxicity.

Ethanolamine is not typically included in serum supplemented media. Murakami's (H. Murakami, et al., Proc. Natl. Acad. Sci. USA, 1982, 79:1158-1162) demonstrates that ethanolamine at 20 uM is stimulatory to growth of a murine hybridoma cell line. Tharakan, et al., J. Immunol. Methods, 1986, 94:225-235; Cole, et al., J. Immunol. Methods, 1987, 97:29-35; and Kovar, et al., Immunol. Letters, 1984, 7:339-345 also found ethanolamine to be stimulatory to growth of several murine hybridoma cell lines. None of these researchers combined elevated levels of ethanolamine with elevated levels of glutamine, choline, tryptophan and other amino acids as we have found necessary for optimum product expression. Several other serum-free media do not include ethanolamine (T. Chang, et al., J. Immunol. Methods, 1980, 39:369-375) and a review by Iscove (N.N. Iscove, Culture of Lymphocytes and Hemopoietic Cells in Serum-Free Medium p. 169-185 in Methods for Serum-Free Culture of neuronal and Lymphoid Cell Lines, Alan R. Liss Inc., NY 1984) identifies choline and inositol as essential, but does not mention ethanolamine. We have found that ethanolamine is effective when supplemented to a level of approximately 1-10 mg/L, and can be included at up to at least 10 mg/L without apparent toxicity.

It should be understood that choline and ethanolamine can be provided in various forms including phosphocholine and phosphoethanolamine or phosphatidylcholine and phosphatidylethanolamine. The relative effectiveness of these various forms will depend on the ability of the specific cell line to take up (transport) and metabolize the complex forms.

Supplementation of standard media with phospholipid precursors alone is not sufficient to achieve the desired maximum extension of culture longevity and product production. Rather, phospholipid precursors at in synergy with the other components described.

c) Tryptophan:

Tryptophan is recognized as an essential amino acid and is included in typical serum-supplemented and serum-free media at 2-20 mg/L, with the exception

of Waymouth's MB752/1 medium for L-929 cells where it is present at 40 mg/L. Several researchers have attempted to optimize media by supplementation with increased levels of each amino acid added separately. Typical of these, Barns and Iscove (N.N. Iscove, Culture of Lymphocytes and Hemopoietic Cells in Serum-Free Medium p. 169-185 in Methods for Serum-Free Culture of Neuronal and Lymphoid Cells) have both tested supplementation of media with each of the standard amino acids, and neither found addition of tryptophan at greater than standard levels to be stimulatory. We have found that tryptophan supplementation is essential to achieving the desired increase in culture longevity and product titre. For optimal effect, tryptophan is supplemented to levels higher than those typically taught in the literature, or greater than 20 mg/L, and can be added at up to at least 100 mg/L without toxic effect.

Supplementation of standard media with tryptophan alone is not sufficient to achieve the desired effect. Rather, tryptophan at elevated concentrations acts in synergy with the other components of the Primary Supplement to achieve the desired maximum extension of culture growth and longevity, and product production.

d) Other Amino Acids:

The recommended Primary Supplement also includes a formulation of amino acids determined to be desirable for expression of a product form a particular cell line.

Amino acids are the essential building blocks for protein synthesis. Supplementation of medium with the components specified about provides the basis for increased culture longevity, and hence, in a significantly increased period for product production. Under these conditions, amino acids required for cell maintenance and product synthesis can become depleted. To maximize final product titre, the levels of these amino acids must be increased so as to not become limiting. Amino acid analysis of spent medium using techniques which are known to the analytical chemist (D.H. Speckman, et al., Automatic Recording Apparatus for Use in the Chromatography of Amino Acids, Analytical Chemistry, 1958, 30:1190-1206) can be used as a tool to identify those amino acids which are depleted during culture and require supplementation.

Iscove (N.N. Iscove, Culture of Lymphocytes and hemopoietic Cells in

Serum-Free Medium p. 169-185 in Methods for Serum-Free Culture of Neuronal and Lymphoid Cells) and others have tested supplementation of media with each of the standard amino acids. Typical of the literature, Iscove found that addition at greater than standard levels of all amino acids tested, (except cystine), was not stimulatory.

Luan (Y.T. Luan, et al., Strategies to Extend Longevity of Hybridomas in Culture and Promote Yield of Monoclonal Antibodies) describes a fed batch strategy, consisting of adding a supplement containing glutamine, essential amino acids (including tryptophan), vitamins (including choline) and serum at various time points during the culture of a murine hybridoma cell line. The medium to which additions was made consisted of DMEM supplemented with fetal calf serum. This method resulted in an increase in culture longevity and increase in final antibody titre. Similarly, Birch (J.R. Birch, et al., Animal Cell Culture, EPA PCT/GB6/00383, 1986) describes fed-batch culture adding a supplement containing glucose, glutamine essential and non-essential amino acids (including tryptophan) during the culture of another murine hybridoma cell line. The medium to which additions was made was also DMEM supplemented with fetal calf serum.

It is important to note regarding Luan and Birch that neither included ethanolamine in their media which we have found to be highly favored phospholipid precursors for the optimum cell growth and product production effect. Further, while we have found that our supplement can be used effectively in fed-batch and continuous culture, we have found (unlike Luan and Birch) that inclusion of the supplement at the start of culture is also effective (i.e. in contrast to Luan and Birch, nutrient addition during culture is not obligatory using the supplement of the current invention). Additionally, applicants' supplement can be used advantageously with serum-free media. Also differentiating the current work, neither Luan and Birch included a defined reducing agent, such as MTG which we have identified, as discussed below, as a preferred embodiment which can result in a further increase in cell product expression.

Class I Reagents

The reagents of the Primary Supplement can be combined with additional reagents to produce media having more favorable cell growth and product expression properties. These, or Class I reagents, include, but are not limited to, reducing

agents, trace metal ions and/or vitamins. Components of the Class I reagents can be added individually or in combination with the complete set of reagents of the Primary Supplement. The utility of these additional Class I reagents will vary depending on the cell line and basal medium composition.

a) Reducing Agents:

The metabolism of glutathione and related sulfhydryl species has been reviewed by Meister (A. Meister, Selective Modification of Glutathione Metabolism, Science, 1983, 220:472-477). Yamane (I. Yamane, et al., Effects of Sulfhydryl Groups and Oxygen Tension on the Cell Proliferating Activity of Bovine Serum Albumin in Culture, Cell Structure and Function, 1982, 7:133-143) shows that addition of reducing agents can protect cultures from damage associated with high oxygen tension.

Some serum-supplemented media (such as RPMI 1640) contain added glutathione (GH) at 1-15 mg/L. Other reducing agents are not generally included in serum-supplemented media. For example, beta-mercaptoethanol (B-ME) and monothioglycerol (MTG) are not typically included in cell culture media, and are not included in any of the media tabulated by Freshey above. In contrast, glutathione or dithiothreitol (DTT) is included in MCDB110 serum-free medium, but are absent from several other serum-free formulations (CDB 170, MCDB 153, WAJC, HITES, etc.). Iscove's serum-free medium does not contain GH or DTT.

It is important to note that although there are reports of media containing reducing agents, that none of these media contain all of the reagents of the Primary Supplement. For instance, Iscove (N.N. Iscove, Culture of Lymphocytes and Hemopoietic Cells in Serum-Free Medium, p 169-185, in Methods for Serum-Free Culture of Neuronal and Lymphoid Cells) recommends supplementation of his IMDM medium with either B-ME (5×10^{-5}M) or MTG 7.5×10^{-5}M. Iscove's medium does not, however, contain ethanolamine or the elevated levels of glutamine, tryptophan and other amino acids required for the synergistic effect observed for cell growth and product expression attributed to reagents of the Primary Supplement. Further, Kawamoto (T. Kawamoto, Anal. Biochem., 1983, 130:445-453) has supplemented a basal medium with several components including ethanolamine (10 μM) and B-ME (10 μM). Kawamoto's media do not, however, contain the elevated levels of

glutamine, tryptophan and other amino acids required for the synergistic effect. Likewise, Kovar (J. Kovar, et al., Immunol. Letters, 1984, 7:339-345) developed a serum-free medium containing ethanolamine (20 μM), but this medium again contains low levels of glutamine, tryptophan and other amino acids and does not result in the synergy which we have discovered. Kovar (J. Kovar, et al., Serum-Free Medium for Hybridoma and Parental Myeloma Cell Cultivation: A Novel Composition of Growth Supporting Substances) tested β-mercaptoethanol as a possible component in their ethanolamine supplemented RPMI-1640 based serum-free medium. This medium did not contain elevated levels of glutamine, tryptophan, other amino acids, or choline, which we have found favors the optimum synergistic effect. In the absence of these components, Kovar and Frank found B-ME not to be useful and deleted it from their final formulation.

We have found that supplementation of culture media with a reducing agent/sulfhydryl compound can result in an increase in culture longevity and product titre. However, such supplementation is maximally effective when in combination with the complete Primary Supplement. Reducing agent/sulfhydryl compounds for use include β-mercaptoethanol, monothioglycerol (MTG), dithiothreitol, glutathione, thioglycolate, and cystine. More preferred are thiol molecules which have hydroxyl group(s) such as (β-mercaptoethanol) B-ME, (monothioglycerol) MTG and (dithiothreitol) DTT. Most preferred are mono-thiol compounds of this class such as B-ME and MTG. These are effective at concentrations above 0.1 mg/L and are not toxic at up to at least 10 mg/L. A preferred concentration is 0.5 - 5 mg/L.

b) Metal Ions:

Metal ions are essential for animal cell culture and are included in typical media as components of salt and trace element mixtures, or as components of undefined supplements such as serum (K. Higuchi, "Cultivation of Animal Cells in Chemically Defined Media - A Review", Advan. Appl. Microbiol., 16:111-136). In media supplemented with the reagents of the Primary Supplement, high cell densities can be reached such that availability of certain metal ions, if included only at the levels incorporated in standard media designed to support lower cell densities, can become limiting. Therefore, in a preferred embodiment of the current invention, metal ions are also included in the supplement as found to be necessary for a

particular cell line. At the high concentrations that can be required, solubility limits of some metal ions may limit the maximum concentration that can be added with beneficial effect. Therefore, a further preferred embodiment is to supply necessary metal ions along with a suitable chelating agent. The chelating agent must be non-toxic at the concentrations added, and must bind the required metal ions in a reversible manner such that they may be held in solution at adequate concentration, but will be delivered to the cells in an active form.

Analysis of the spent medium using techniques which are well known to the analytical chemist ("Flame Photometry", Chpt. 11, in H. Willard, et al., Instrumental Methods of Analysis, Van Nostrand Co., 1965) can be used as a tool to identify those metal ions which are utilized and may require supplementation. Some metal ions which may require supplementation include, but are not limited to, calcium, magnesium, molybdenum, cobalt, copper, manganese, zinc, selenium and iron. A more extensive list is given by Hamilton and Ham (W. Hamilton, et al., Clonal Growth of Chinese Hamster Cell Lines in Protein-Free Media, In Vitro, 1977, 13(9):537-547).

Various metal chelators have been used in cell culture. Natural proteins which can serve this function include transferrin and ferritin, especially to chelate iron, and albumin to chelate a variety of multivalent metal ions. Some other chelators which have been used especially to chelate iron include pyridoxyl isonicotinoyl hydrazone, choline citrate, citrate (C. III, T. Brehm, et al., Species Specificity of Iron Delivery in Hybridomas, In Vitro Cell. Develop. Biol., 1988, 24(5):413-419) and acetylacetonate (L. Rasmussen, et al., Utilization of Iron Complexes in an Animal Cell, J. Cellular Physiol., 1985, 122:155-158). We have found citrate at about 1-10 mM to be an effective chelator capable of supplying several multivalent metal ions. In addition, a variety of other organic acids such as malic acid, succinic acid, fumaric acid and alpha ketoglutaric acid are effective chelators.

c) Vitamins:

Vitamins are essential for animal cells in culture and are generally included in cell culture media (K. Higuchi, Cultivation of Animal Cells in Chemically Defined Media - A Review p. 111-136 in Advan. Appl. Microbiol., 1973, 16:111). In media

supplemented only with the Primary Supplement composition, high cell densities can be reached such that availability of certain vitamins, if included only at the levels incorporated in standard media designed to support lower cell densities, can become limiting. Therefore, in a further preferred embodiment of the current invention, vitamins may also be included in the supplement as found to be necessary for a particular cell line. Vitamins which may become limiting include, but are not limited to, p-amino-benzoic acid, biotin, folic acid, folinic acid, nicotinamide, pantothenate, pyridoxine, riboflavin, flavin adenine dinucleotide, ascorbic acid, thiamine and vitamin B-12.

Having generally described the media supplements of the instant invention, several limitations associated with their use, as well as ways to circumvent the limitations warrant discussion.

First, although it is convenient to define classes of reagents that can be readily combined with culture media that is fed to cells without later during the culture period having to refeed the cells, it will be appreciated by those skilled in the art that similar favorable effects can be gained by adding individual reagents to the culture media during the culture period, so as to maintain their concentrations at the levels provided by the supplements. This is most apparent with regard to glutamine, which can be added in combination with the other reagents of the Primary Supplement, or omitted and individually added and maintained at the desired level.

Secondly, the concentrations of reagents that are used in the various supplements will vary over the ranges stated, and are a function of both the cell culture media to which the supplements are added, as well as the particular cell type cultured. The optimal concentrations of the reagents are readily determined by those skilled in the art.

Thirdly, with the exception of reagents of the Primary Supplement, all of which must be present in the supplements for maximum cell culture benefit, only one of the Class I reagents need be present for advantageous results.

Fourthly, it will be realized that for optimal results, the basal medium to which the supplement is added must be appropriate for the cell line of interest, with key nutrients available at adequate levels to enhance cell growth or product expression. Thus, for example, it may be necessary to increase the level of glucose (or other energy source) in the basal medium, or to add glucose (or other energy

source) during the course of culture, if this essential energy source is found to be depleted and to thus limit cell growth or product expression.

The following examples illustrate the invention, but are not intended to limit it in any manner. For instance, hybridomas and antibodies are illustrative of cell lines that can be successfully grown and harvested, respectively in media supplemented with the instant compositions. However, such media are not limited to growing hybridomas or harvesting antibody, but rather can be used to grow a side variety of cells that produce a broad range of products.

Example 1

A Representative Commercial Medium Formulation

Ventrex HL-1 is a medium, representative of state-of-the-art commercially available media for cell culture. Product literature indicates that this medium "was designed for use in the culture of hybridomas and lymphoid cells". The medium is claimed to be effective for growth of many transformed and established cell lines, and especially for production of monoclonal antibodies from murine and human hybridomas. A list of 55 cell lines grown successfully in this medium is presented in Current Concepts, 1(1):5, published by Ventrex, 217 Read St., Portland, Maine.

Figure 1 shows the growth and product expression from a hybridoma cell line, D-234, producing a human monoclonal antibody in HL-1 medium. The maximum viable cell density of 1.3 x 10^6 cell/ml is reached at 120 hours and the culture dies rapidly thereafter. The maximum total cell density is 2 x 10^6 cells/ml. The final antibody titre is 70 mg/L.

The hybridoma D-234 is on deposit with the American Type Culture Collection with Accession No. HB 8598.

The hybridoma produces an IgM antibody which was assayed using standard ELISA Techniques.

Example 2

Serum-Free Hybridoma Medium Formulation

Table I, column 1, shows a typical serum-free medium formulation (50% RPMI, 50% DME, bovine insulin (5 mg/L), human transferrin (5 mg/L), selenium (5 µg/L), 0.1% Pluronic polyol (F68). This medium is similar to several

compositions reported in the literature.

Figure 2 shows growth and product expression by the D234 hybridoma in the medium of Table I, column 1. Growth and product expression are similar to that in the commercially available HL-1 medium. The maximum viable cell density of 1.3 x 10^6 cells/ml. The final antibody titre is 80 mg/L.

Example 3

Medium Containing Primary Supplement Addition Of Glutamine Before And During The Culture Period

Table I, column 4, shows a composition wherein the standard medium has been supplemented with the reagents of the Primary Supplement (glutamine at 8 mM, tryptophan at 140 mg/L, choline at 20 mg/L, ethanolamine at 10 mg/L and other amino acids increased in concentration by typically around 3 fold the concentration in the basal medium). In initial studies it was determined that the glucose level in the basal medium could be depleted and limit culture growth and longevity. Therefore, in addition to being included in the basal medium, additional glucose was added during the course of the culture period. Further, glutamine, in addition to being incorporated in the Primary Supplement to the medium at the start of culture, was also added at day 4, 8 and 12 such that the total glutamine supplied was equivalent to 20 mMoles per litre of culture.

Figure 3 shows growth and product expression by D234 in the supplemented medium. Maximum viable cell density of 1.2 x 10^6 cell/ml is reached at 120 hours, thereafter the culture is maintained in a pseudo-stationary phase, where cell growth approximately balances cell death such that the viable cell concentration declines only slowly over a period for 100 hours. The total cell density continues to rise to 2.5 x 10^6 cells/ml. Cells continue to express product over the pseudo-stationary phase, and a final titre of 290 mg human antibody per litre is reached.

Example 4

Medium Containing Primary Supplement Addition Of Glutamine At The Start Of The Culture Period Only

D234 was grown in medium and conditions similar to those in Example 3, except that glutamine was included in the Primary Supplement to make the concentration of glutamine 20 mM at the start of culture, and glutamine was not

added thereafter. Growth and product expression were equivalent to those in Example 3.

Example 5

Medium Containing Primary Supplement and Class I Reagent

Table I, column 2, shows a composition wherein standard medium has been supplemented with the reagents of the Primary Supplement as well as with monothioglycerol (a Class I reagent) at 10 mg/L. Glucose and glutamine were supplied as in Example 3.

Figure 4 shows growth and product expression by D234 in the supplemented medium. Maximum viable cell density of 1.9 x 10^6 cell/ml is reached at 120 hours. Thereafter, the culture is maintained in a pseudo-stationary phase, where cell growth rate approximately balances the rate of cell death such that the viable cell concentration declines only slowly over a period of 150 additional hours. Total cell density continues to climb to 3.8 x 10^6 cells/ml. Cells continue to express product over the pseudo-stationary phase period and a final titre of 230 mg human antibody per L is reached.

Example 6

Supplement Used In Combination With Lipid Emulsion

Table I, column 3, shows a composition in which the typical cell culture medium has been supplemented with the reagents of the Primary Supplement (8 mM glutamine, 28 mg/L tryptophan, 22.8 mg/L choline, 0.8 mg/L ethanolamine, and other amino acids supplemented to typically around 3 fold the basal medium concentration), and with Class I reagents (1 mg/L monothioglycerol, vitamin B-12 and trace elements including molybdenum, cobalt, copper, zinc and iron). Additionally, the medium contains lipids (linoleic acid, tween 80, lecithin, cholesterol and vitamin E). Additionally, glucose and glutamine was added during the culture period as described in Example 3.

Figure 5 shows the growth and product expression by D234 in the supplemented medium. Maximum viable cell density of 1.7 x 10^6 cells/ml is reached at 80 hours. Thereafter, the culture is maintained in a pseudo-stationary phase during which growth continues at approximately 50% of the maximum exponential phase

growth rate which approximately balances the rate of death such that the viable cell concentration declines only slowly over a period of 150 additional hours. Total cell density continues to climb to 3.5 x 10^6 cells/ml. Cells continue to express product over the pseudo-stationary phase period and a final titre of 275 mg human antibody per L is reached.

Example 7

Use Of Supplement In Fed-Batch Culture

The supplement of the present invention can be used beneficially in fed-batch, as well as in simple batch culture. Table I, column 5, shows the standard medium supplemented with reagents of the Primary Supplement and Class I reagents, as well as with lipids. This medium is similar to that of Example 6 (Table I, column 3).

This medium was used for growth of D234 in a fed-batch process. After 120 hours, additional supplement was added as shown in Table II. After 220 hours, glutamine was further supplemented with another 5 mmoles/L of culture.

Figure 6 shows the growth and product expression by D234 in the supplemented fed-batch culture. Maximum viable cell density of 1.8 x 10^6 cells/ml is reached at 130 hours. Thereafter, the culture is maintained in a pseudo-stationary phase during which growth continues at approximately 20% of the maximum exponential phase growth rate which approximately balances the rate of death such that the viable cell concentration declines only slowly over a period of 190 additional hours. Total cell density continues to climb to 3.7 x 10^6 cells/ml. Cells continue to express product over the pseudo-stationary phase period and a final titre of 470 mg human antibody per L is reached.

Other feeding strategies have also been tested wherein the nutrients are added to a similar total final concentration as in the above sample, but are added at different schedules. Subdivision of the supplement addition into nearly equal daily additions beginning after 190 hours gives results similar to those described above. Slow continuous feeding also gives similar results.

Having described what applicants believe their invention to be, it will be appreciated that the invention should not be construed as limited in any manner whatsoever other than by the scope by the appended claims.

TABLE 1 (mg/l)

		1	2	3	4	5
1.	CaCl2	0.0000	0.0000	400.0000	100.0000	200.00000
2.	Ca(No3)2.4H20	50.0000	50.0000	40.0000	50.0000	45.00000
3.	Fe(NO3)3.9H20	0.0500	0.0500	0.0400	0.0500	0.04500
4.	KCl	400.0000	400.0000	560.0000	400.0000	480.00000
5.	MgSO4	73.5000	73.5000	242.8000	173.5000	158.15000
6.	NaCl	6000.0000	6000.0000	5300.0000	6000.0000	5650.00000
7.	NaH2PO4.H20	625.0000	625.0000	732.0000	625.0000	678.50000
8.	Na2HPO4	400.5000	400.5000	320.4000	400.5000	360.45000
9.						
10.	Glucose	2800.0000	3800.0000	2700.0000	4800.0000	2700.00000
11.	Glutathione	0.5000	0.5000	0.4000	0.5000	0.45000
12.	HEPES	2979.0000	2979.0000	2383.2000	2979.0000	2681.10000
13.	Na Pyruvate	55.0000	55.0000	44.0000	1155.0000	49.50000
14.	NaHCO3	2850.0000	2850.0000	2350.0000	2850.0000	2600.00000
15.	Phenol Red	10.0000	10.0000	8.0000	10.0000	9.00000
16.						
17.	pAmino Benzoic Acid	0.5000	0.5000	0.4640	1.0000	0.48200
18.	Biotin	0.1000	0.1000	0.1120	.2000	0.10600
19.	Ca Pantothenate	2.1250	2.1250	1.7016	4.0000	1.91330

TABLE I(mg/l)

Continued

	1	2	3	4	5
20. Folic acid	2.5000	2.5000	1.6000	4.0000	1.80000
21. Nicotinamide	2.5000	2.5000	2.0000	5.0000	2.25000
22. Pyridoxal HCl	2.0000	2.0000	1.6000	4.0000	1.80000
23. Pyridoxine HCl	0.5000	0.5000	0.4800	1.0000	0.49000
24. Riboflavin	0.3000	0.3000	0.2560	.6000	0.27800
25. Thiamine HCl	2.5000	2.5000	2.0160	5.0000	2.25800
26. Vitamin B12	0.0025	0.0025	0.0500	.2400	0.02625
27.					
28. Choline Chloride	3.5000	23.5000	22.8000	23.5000	23.15000
29. Inositol	21.0000	41.0000	24.8800	41.0000	27.94000
30.					
31. Ethanolamine	0.0000	10.0000	0.8	10.0000	0.9
32. Glycerol	0.0000	200.0000	0.0000	200.0000	180.00000
33.					
34. Glutamine	1160.0000	1160.0000	1160.0000	1160.0000	1180.00000
35. Pluronic Polyol F68	1000.0000	1000.0000	1000.0000	1000.0000	1000.00000
36.					
37. Insulin	5.0000	5.0000	4.0000	5.0000	4.50000

TABLE 1 (mg/l)

Continued

	1	2	3	4	5
38. Transferrin	5.0000	5.0000	4.0000	5.0000	4.50000
39.					
40. Na2SeO3	0.0000	0.0050	0.0040	0.0050	0.00450
41. FeCl3	0.0000	0	0.0000	1.6000	0
42. NH4Mo7024.4H20	0.0000	0	0.0080	0.1000	0.00400
43. CoC12.6H20	0.0000	0.1000	0.0100	0.1000	0.00500
44. CuC12.2H20	0.0000	0	0.0400	0.1000	0.02000
45. MnC12.4H20	0.0000	0.1000	0.0040	0.1000	0.00200
46. ZC12	0.0000	0	0.0080	0.1000	0.00400
47.					
48. Fes04.7H20	0.0000	0.0000	0.1100	0.0000	0.05500
49.					
50. Niacin	0.0000	0	0.0320	0.0000	0.01600
51. Hydroxyproline	0.0000		160.0000	0.0000	80.00000
52. Beta Alanine	0.0000		60.0000	0.0000	30.00000

TABLE 1 (mg/l)
Continued

	1	2	3	4	5
53. Malic acid	0.0000		10.7200	0.0000	5.36000
54. alpha Ketoglutaric acid	0.0000		5.9200	0.0000	2.96000
55. Succinic acid	0.0000		0.9600	0.0000	0.48000
56. Fumaric acid	0.0000	0	0.8800	0.0000	0.44000
57. D Serine	0.0000	0	40.0000	0.0000	20.00000
58.					
59.					
60.					
61. sucrose	0.0000		330.0000	0.0000	165.00000
62. maltose	0.0000		200.0000	0.0000	100.00000
63.					
64. Ethanol	0.0000	0.0000	640.0000	0	720.00000
65. Tween 80	0.0000	0.0000	1.6000	0	1.80000
66. Lecithin	0.0000	0.0000	0.8000	0	0.90000
67. Linoleic acid	0.0000	0.0000	0.8000		0.90000
68. Cholesterol	0.0000	0.0000	0.4000		0.45000
69. alpha Tocopherol acetate	0.0000	0.0000	0.4000	0	0.45000
70.	0.0000				

TABLE 1 (mg/l)

Continued

	1	2	3	4	5
71. Monothioglycerol	0.0000	0.0000	0.8000	10.0000	0.90000
72. Glucose citrate				258.0000	

TABLE 1 (mg/l)
Continued

	1	2	3	4	5
1. ARG	142	542	246.2	794	194.1
2. ASP	25	125	280.0	125	152.5
3. ASP AC.	10	50	274.0	50	142.0
4. CYSTINE	49	49	59.2	195	54.1
5. GLUT.AC.	10	50	308.0	50	159.0
6. GLY	20	40	56.0	40	38.

TABLE 1 (mg/l)
Continued

	1	2	3	4	5
7. HIS	28	58	62.4	120	45.2
8. ISOLEU	78	178	212.4	282	145.2
9. LEU	78	178	112.4	282	95.2
10. LYS. HCL	93	173	214.4	317	153.7
11. MET	22	52	217.6	82	119.8
12. PHENYLALA	40	70	232.0	136	136.0
13. PRO	10	50	108.0	50	59.0
14. SER	36	136	68.8	96	52.4
15. THREO	58	98	86.4	194	72.2
16. TRP	10	120	28.0	140	19.0
17. TYRO	46	146	86.8	158	66.4
18. VAL	57	97	145.6	191	101.3

TABLE II

1.	CaCl2	400.0000
2.	Ca(No3)2.4H20	0.0000
3.	Fe(NO3)3.9H20	0.0000
4.	KCl	240.0000
5.	MgSO4	184.0000
6.	NaCl	500.0000
7.	NaH2PO4.H20	232.0000
8.	Na2HPO4	0.0000
9.		
10.	Glucose	500.0000
11.	Glutathione	0.0000
12.	HEPES	0.0000
13.	Na Pyruvate	0.0000
14.	NaHCO3	0.0000
15.	Phenol Red	0.0000
16.		
17.	pAmino Benzoic Acid	0.0640
18.	Biotin	0.3200
19.	Ca Pantothenate	0.0016
20.	Folic acid	0.0160
21.	Nicotinamide	0.0000
22.	Pyridoxal HCl	0.0000
23.	Pyridoxine HCl	0.0800
24.	Riboflavin	0.0160
25.	Thiamine HCl	0.0160
26.	Vitamin B12	0.0480
27.		
28.	Choline Chloride	4.0000
29.	Inositol	0.0800
30.		
31.	Ethanolamine	0.0000

TABLE II
Continued

32.	Glycerol	0.0000
33.		
34.	Glutamine	1660.0000
35.	Pluronic Polyol F68	200.0000
36.		
37.	Insulin	0.0000
38.	Transferrin	0.0000
39.		
40.	Na2SeO3	0.0000
41.	FeCl3	0.0000
42.	NH4Mo7024.4H20	0.0080
43.	CoC12.6H20	0.0100
44.	CuC12.2H20	0.0400
45.	MnC12.4H20	0.0040
46.	ZC12	0.0080
47.		
48.	Fes04.7H20	0.1100
49.		
50.	Niacin	0.0320
51.	Hydroxyproline	160.0000
52.	Beta Alanine	60.0000
53.	Malic acid	10.7200
54.	alpha Ketoglutaric acid	5.9200
55.	Succinic acid	0.9600
56.	Fumaric acid	0.8800
57.	D Serine	40.0000
58.		
59.		
60.		

TABLE II

Continued

61.	sucrose	330.0000
62.	maltose	200.0000
63.		
64.	Ethanol	0.0000
65.	Tween 80	0.0000
66.	Lecithin	0.0000
67.	Linoleic acid	0.0000
68.	Cholesterol	0.0000
69.	alpha Tocopherol acetate	0.0000
70.		
71.	Monothioglycerol	20.0000
72.	Glucose	500.0000

TABLE II

Continued

1. ARG	132.6
2. ASP	260.0
3. ASP AC.	266.0
4. CYSTINE	20.0
5. GLUT.AC.	300.0
6. GLY	40.
7. HIS	40.0
8. ISOLEU	150.0
9. LEU	50.0
10. LYS. HCL	140.0
11. MET	200.0
12. PHENYLALA	200.0
13. PRO	100.0
14. SER	40.0
15. THREO	40.0
16. TRP	20.0
17. TYRO	50.0
18. VAL	100.0

WE CLAIM:

1. A cell culture medium Primary Supplement comprising glutamine or glutamate, amino acids and phospholipid precursors, in amounts that effectively maintain cells in culture for prolonged times in a pseudo-stationary growth phase.

2. The cell culture medium Primary Supplement as described in claim 1, wherein said phospholipid precursors are selected from the group consisting of serine, inositol, choline, ethanolamine, and glycerol.

3. The cell culture medium Primary Supplement as described in claim 2, wherein the phospholipid precursors comprise both ethanolamine and choline.

4. A cell culture medium Primary Supplement as described in claim 1, wherein said phospholipid precursors are supplied in complex form.

5. A cell culture medium Primary Supplement as described in claim 1, wherein said phospholipid precursors supplied in complex form are selected from the group consisting of phosphoethanolamine, phosphocholine, phosphatidylcholine, and phosphatidylethanolamine.

6. The cell culture medium Primary Supplement as described in claim 5, wherein said amino acids comprise tryptophan at a concentration that when added to culture media is more than about 20 mg/L, but less than about 200 mg/L.

7. The cell culture medium Primary Supplement as described in claim 6, wherein the glutamine or glutamate that when added to culture media is above about 5 mM, but below about 40 mM.

8. The cell culture medium Primary Supplement as described in claim 7, wherein choline is present at a concentration that when added to culture medium is greater than about 4 mg/L, but less than about 50 mg/L.

9. The cell culture medium Primary Supplement as described in claim 8, wherein ethanolamine is present at a concentration greater than about 1 mg/L, but less than about 100 mg/L.

10. Cell culture media comprising the Primary Supplement of claim 9.

11. The cell culture media of claim 10 further comprising growth hormones, growth factors or serum.

12. Cell culture medium supplement comprising said Primary Supplement of claim 1 and Class I reagents, said Class I reagents present in effective amounts that co-act with said Primary Supplement to maintain cells in a pseudo-stationary phase, and selected from the group consisting of reducing agents, trace metal ions and vitamins.

13. Cell culture medium supplement as described in claim 12, wherein said reducing agents are selected from the group consisting of beta-mercaptoethanol, monothioglycerol and dithiothreitol.

14. Cell culture medium supplement as described in claim 13, wherein said reducing agent is included at a concentration greater than about 0.1 mg/L and less than about 100 mg/L.

15. Cell culture media comprising the supplement of claim 12.

16. Cell culture media of claim 13 further comprising growth hormones, growth factors or serum.

17. Cell culture medium supplement as described in claim 12, wherein said trace metal ions are selected from the group consisting of iron, calcium, manganese, magnesium, molybdenum, cobalt, copper, zinc, and selenium.

18. Cell culture medium supplement as described in claim 17, further

including a chelator.

19. Cell culture medium supplement as described in claim 18, wherein said chelator is selected from the group consisting of transferrin, ferritin, albumin, pyridoxyl isonicotinoyl hydrazone, choline citrate, and citrate.

20. Cell culture medium supplement as described in claim 19, wherein said chelator is citrate and is included in medium at a concentration of about 1-10 mM.

21. Cell culture media comprising the supplement of claim 20.

22. Cell culture media of claim 21 further comprising growth hormones, growth factors or serum.

23. Cell culture medium supplement as described in claim 12, wherein said vitamins are selected from the group consisting of p-amino-benzoic acid, biotin, folic acid, folinic acid, nicotinamide, pantothenate, pyridoxine, riboflavin, flavin adenine dinucleotide, ascorbic acid, thiamine and vitamin B-12.

24. Cell culture media comprising the supplement of claim 21.

25. The cell culture media of claim 24 further comprising growth hormones, growth factors or serum.

26. A method of growing cells, comprising contacting said cells with the cell culture medium of claim 10.

27. A method of growing cells, comprising contacting said cells with the cell culture medium of claim 11.

28. The method of claim 26, wherein said cells are antibody secreting cells.

29. A method of growing cells, comprising contacting said cells with cell culture media wherein individually or in combination components of the Primary Supplement of claim 1 are added to said cell culture media over the time of cell culture to maintain said cells in a pseudo-stationary growth phase.

30. A method of growing cells, comprising contacting said cells with cell culture media wherein individually or in combination components of the Primary Supplement and Class I reagents are added to said cell culture media over the time of cell culture to maintain said cells in a pseudo-stationary growth phase.

31. Biochemicals produced by cells grown in the media of claim 10.

32. Biochemicals produced by cells grown in the media of claim 15.

33. Biochemicals produced by cells grown in the media of claim 26.

34. Cell culture medium comprising the Primary Supplement of claim 9 and a lipid emulsion.

35. Cell culture medium comprising the Primary Supplement of claim 34, wherein said lipid emulsion comprises a fatty acid, a nonionic detergent, a phospholipid, an antioxidant and cholesterol.

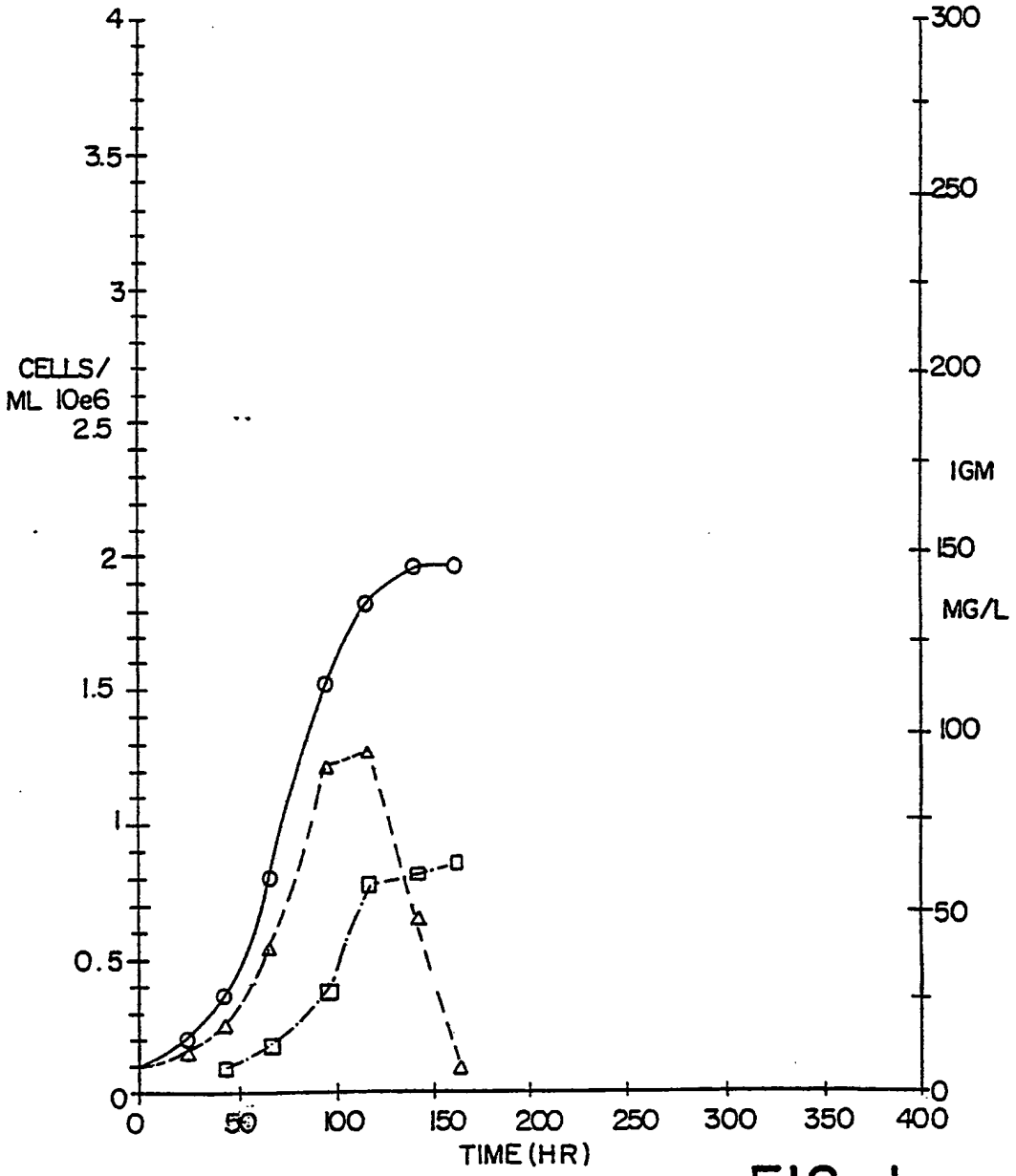
36. Cell culture medium comprising the Primary Supplement of claim 14 and a lipid emulsion.

37. Cell culture medium comprising the Primary Supplement of claim 36, wherein said lipid emulsion comprises a fatty acid, a nonionic detergent, a phospholipid, an antioxidant and cholesterol.

38. Cell culture medium comprising the Primary Supplement of claim 25

and a lipid emulsion.

39. Cell culture medium comprising the Primary Supplement of claim 38, wherein said lipid emulsion comprises a fatty acid, a nonionic detergent, a phospholipid, an antioxidant and cholesterol.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350
400
TIME (HR)
TOTAL CELLS
VIABLE CELLS
IGM

FIG. 1

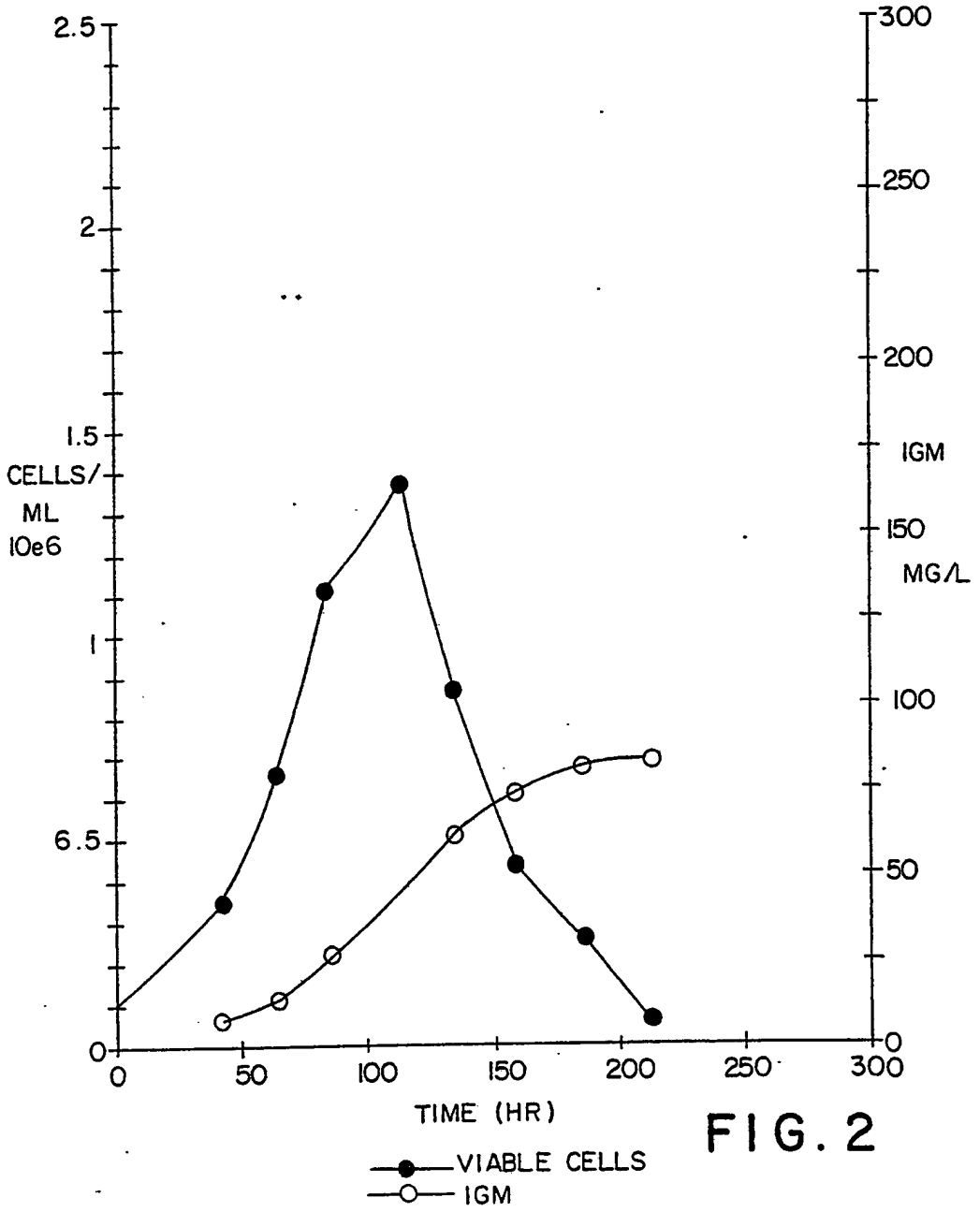
2.5
2
1.5
CELLS/
ML
10e6
1
6.5
0
0
50
100
150
200
250
300
TIME (HR)
300
250
200
IGM
150
MG/L
100
50
0
VIABLE CELLS
IGM

FIG. 2

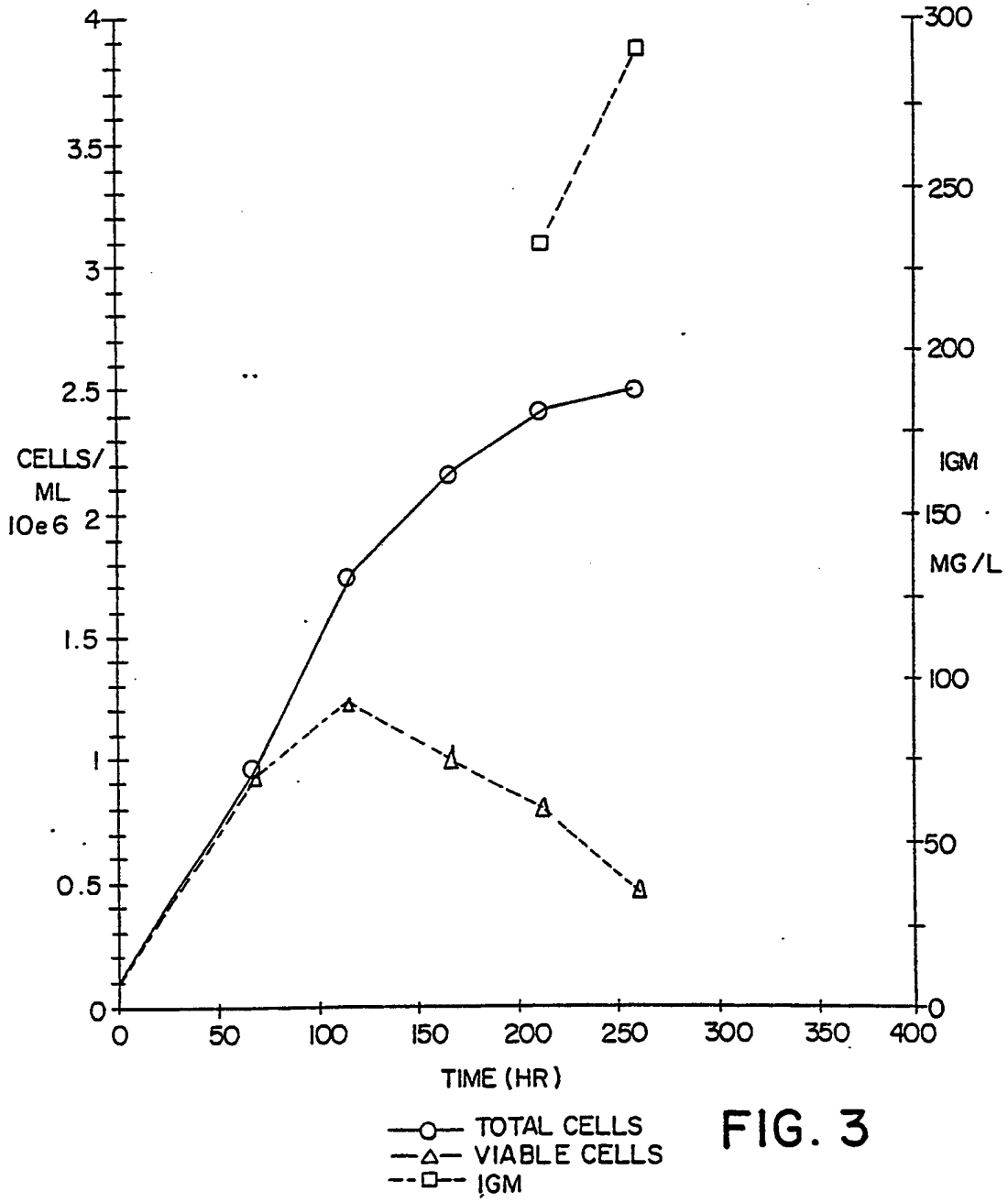
CELLS/ ML 10e6
IGM MG/L
TIME (HR)
0
0.5
1
1.5
2
2.5
3
3.5
4
50
100
150
200
250
300
350
400
TOTAL CELLS
VIABLE CELLS
IGM

FIG. 3

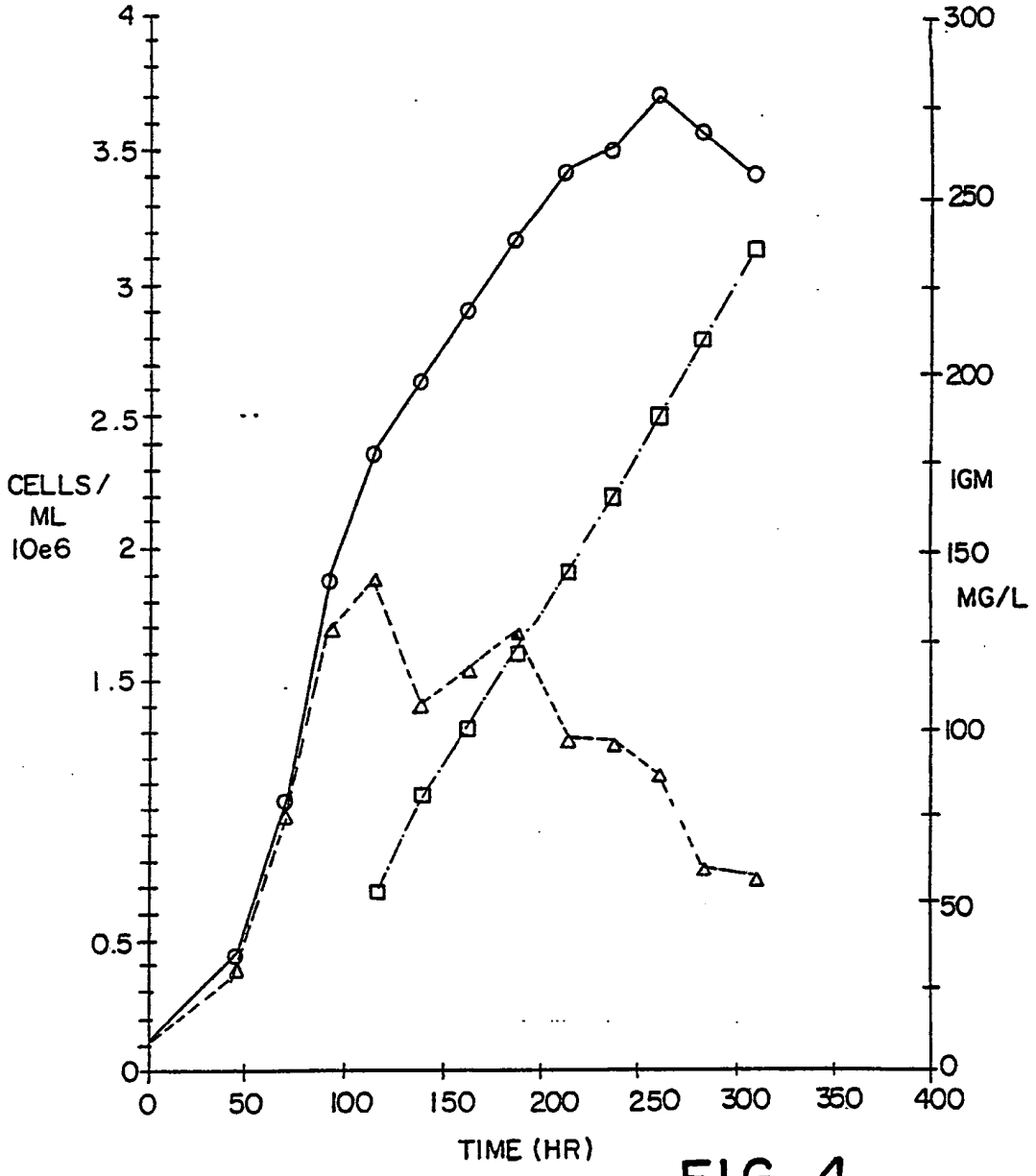
4
3.5
3
2.5
2
1.5
0.5
0
CELLS / ML 10e6
300
250
200
150
100
50
0
IGM
MG/L
0
50
100
150
200
250
300
350
400
TIME (HR)
TOTAL CELLS
VIABLE CELLS
IGM

FIG. 4

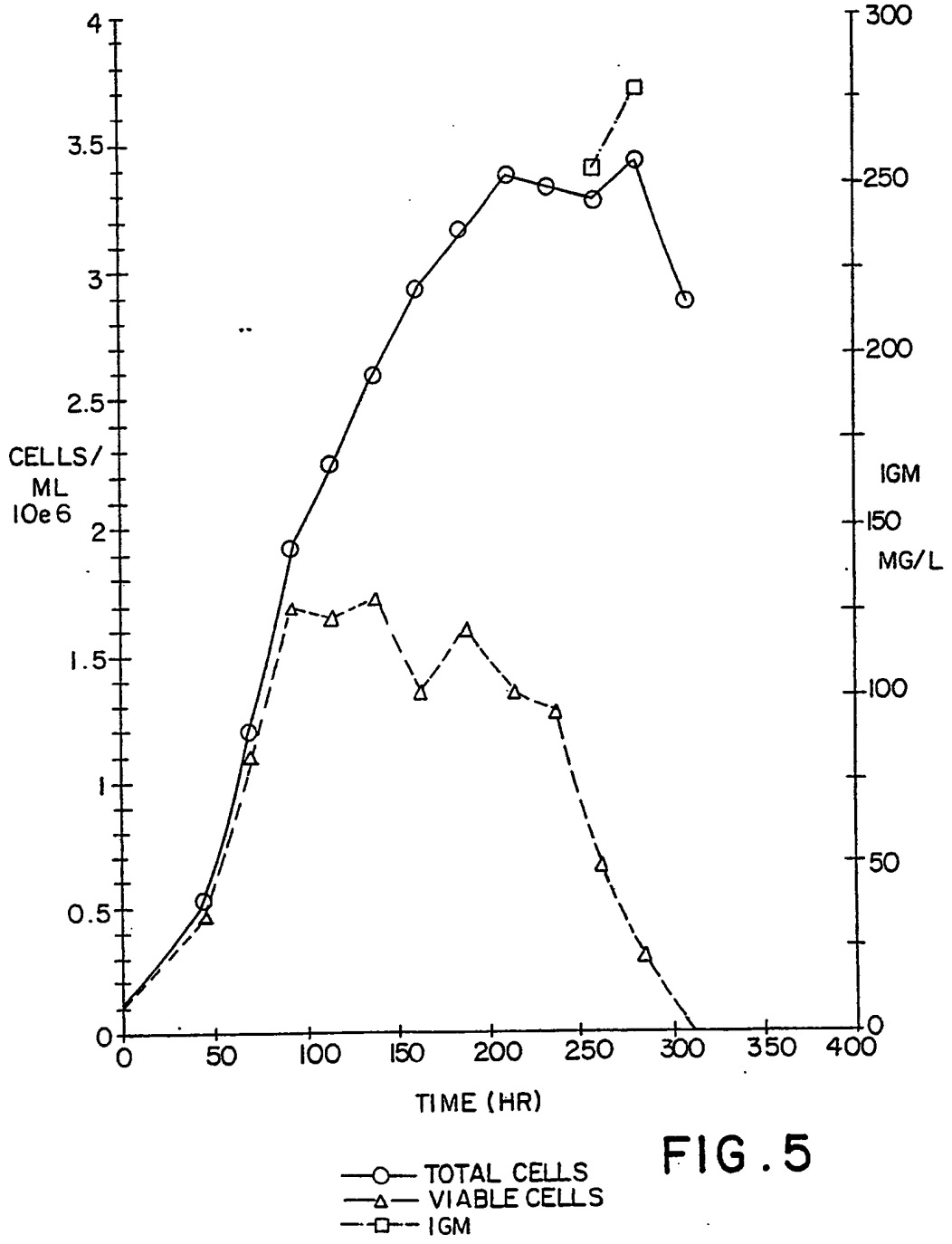

FIG. 5

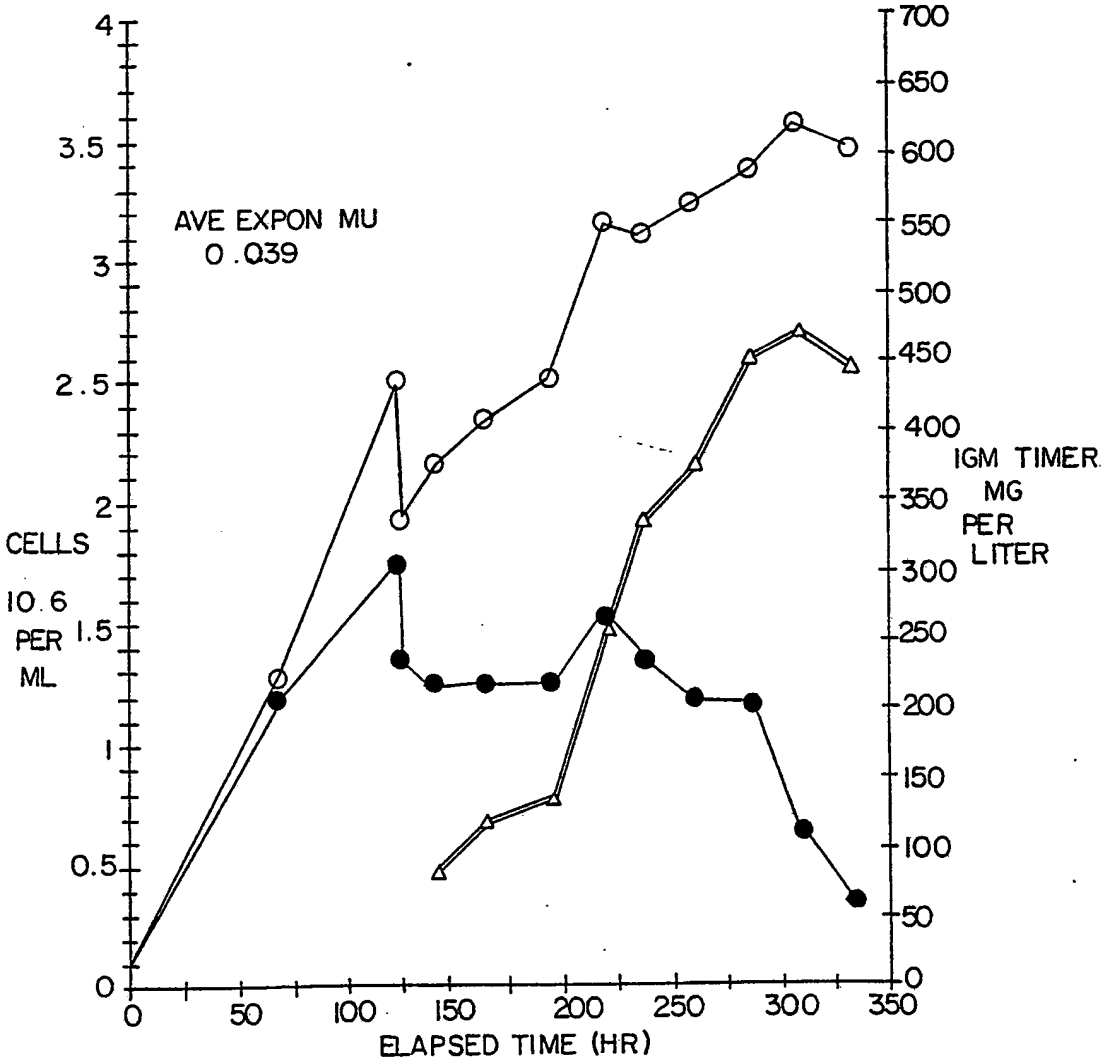
AVE EXPON MU
0.039
CELLS
10.6
PER
ML
IGM TIMER
MG
PER
LITER
ELAPSED TIME (HR)
0
0.5
1
1.5
2
2.5
3
3.5
4
50
100
150
200
250
300
350
400
450
500
550
600
650
700
VIABLE CELLS
TOTAL CELLS
IGM

FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/03986

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 5/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC5	C 12 N 5/00, 5/02

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	WO, A1, 87/00195 (CELLTECH LIMITED) 15 January 1987,	1-33
Y	see pages 7-11, 14-16 and 26-27, fig. 1e and page 27, paragraph.3	1-33
	--	
X	EP, A1, 0 249 557 (FONDATION CENTRE NATIONAL DE TRANSFUSION SANGUINE) 16 December 1987, see pages 3-4 and claims	1-5,9-16,18-28,31-33
	--	
X	US, A, 4 657 866 (S. KUMAR) 14 April 1987, see the whole document	1,2,12-28,31-33
Y		3-11,29-30
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* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
4th December 1989	15. 01. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Biotechnology Letters, Vol. 9, No. 10, September 1987, Y.T. Luan et al.: "Strategies to extend longevity of hybridomas in culture and promote yield of monoclonal antibodies ", see page 691 - page 696 page 693	6
Y	US, A, 4 560 655 (PAUL E. BAKER) 24 December 1985, see table 1 and claim 20	5,8
X	GB, A, 2 196 348 (CESKOSLOVENSKA AKADEMIE VED) 27 April 1988, see claims and example 1	1,2
Y		9
X	Immunology Letters, Vol. 7, 1984, J. Kovár et al.: "Serum-free medium for hybridoma and parental myeloma cell cultivation: a novel composition of growth-supporting substances", see page 339 - page 345 page 340, fig. 1, table I and page 344	1,2
Y		9
A	Journal of Immunological Methods, Vol. 97, 1987, S.P.C. Cole et al.: "Growth of human X human hybridomas in protein-free medium supplemented with ethanolamine ", see page 29 - page 35 the whole document	1-5,9
A	Journal of Immunological Methods, Vol. 94, 1986, John P. Tharakan et al.: "Hybridoma growth and antibody secretion in serum-supplemented and low protein serum-free media ", see page 225 - page 235 page 226, right column, tables I and II, page 231, right column	1,2,9
A	Proc. Natl. Acad. Sci. USA, Vol. 79, February 1982, Hiroki Murakami et al.: "Growth of hybridoma cells in serum-free medium: Ethanolamine is an essential component ", see page 1158 - 1162 the whole document	1-5

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 89/03986

SA 31167

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 87/00195	15/01/87	AU-D-	61348/86	30/01/87
		EP-A-	0229809	29/07/87
		JP-T-	62503146	17/12/87
		GB-A-	2195655	13/04/88
EP-A1- 0 249 557	16/12/87	FR-A-	2600076	18/12/87
		JP-A-	63022183	29/01/88
US-A- 4 657 866	14/04/87	NONE		
US-A- 4 560 655	24/12/85	EP-A-	0112174	27/06/84
		AU-D-	22321/83	21/06/84
		JP-A-	59232090	26/12/84
		CA-A-	1209070	05/08/86
		AU-A-	565932	01/10/87
GB-A- 2 196 348	27/04/88	FR-A-	2604727	08/04/88
		DE-A-	3733453	14/04/88

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